Expression pattern of a butterfly *achaete-scute* homolog reveals the homology of butterfly wing scales and insect sensory bristles

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Background: Lepidopteran wing scales are the individual units of wing color patterns and were a key innovation during Lepidopteran evolution. On the basis of developmental and morphological evidence, it has been proposed that the sensory bristles of the insect peripheral nervous system and the wing scales of Lepidoptera are homologous structures. In order to determine if the developmental pathways leading to *Drosophila* sensory bristle and butterfly scale formation use similar genetic circuitry, we cloned, from the butterfly *Precis coenia*, a homolog of the *Drosophila achaete-scute* (AS-C) genes – which encode transcription factors that promote neural precursor formation – and examined its expression pattern during development.

Results: During embryonic and larval development, the expression pattern of the AS-C homolog, *ASH1*, forecasted neural precursor formation. *ASH1* was expressed both in embryonic proneural clusters – within which an individual cell retained *ASH1* expression, enlarged, segregated, and became a neural precursor – and in larval wing discs in putative sensory mother cells. *ASH1* was also expressed in pupal wings, however, in evenly spaced rows of enlarged cells that had segregated from the underlying epidermis but, rather than give rise to neural structures, each cell contributed to an individual scale.

Conclusions: *ASH1* appears to perform multiple functions throughout butterfly development, apparently promoting the initial events of selection and formation of both neural and scale precursor cells. The similarity in the cellular and molecular processes of scale and neural precursor formation suggests that the spatial regulation of an AS-C gene was modified during Lepidopteran evolution to promote scale cell formation.

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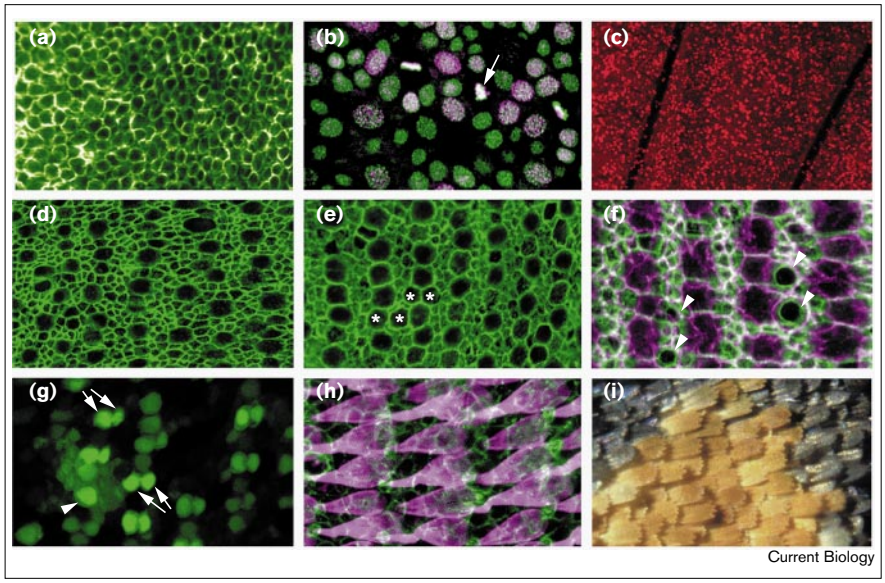
Background

The origin of novel structures during evolution has been difficult to explain. One of the challenges is that partial structures might not have an adaptive value. Co-option, or reuse of a pre-existing ancestral structure in a descendant for a new purpose, is a way to account for intermediate structures [1]. Functional shifts have been suggested for several morphological features, including the use of forearm components during the evolution of vertebrate wings and the modification of reptile scales during the evolution of bird feathers [2]. Morphological [3], cell biological [4–7], and developmental evidence [8] have all been used to propose that insect sensory bristles were co-opted during the evolution of Lepidopteran scales — the flat, striated, and pigmented cuticular evaginations of epithelial cells which are the fundamental units of wing color patterns in butterflies and moths — and thus, that sensory bristles and scales are homologous [3]. In these purported cases of co-option, it has not been shown whether the structures being compared have a similar genetic circuitry.

The Lepidoptera are named for their scale-covered wings, and have both innervated and non-innervated types of scale. Wing sensory scales are located along the veins and the wing margin, and function in mechanosensation and, in some instances, pheromone production [3]. The wing covering comprises mostly non-innervated, structural scales, whose ultrastructure and pigmentation function in thermoregulation [9,10] and color patterning [11].

Details of early structural scale development are best understood from observations made in the moth *Ephestia kühniella* [12,13] and have been extended both by Nijhout [14,15] and our own observations illustrated here (Figure 1). In Lepidoptera, developing wings are set aside as imaginal discs during larval development. Stossberg's [12] analysis of scale cell lineages in pupal wings revealed that a subpopulation of epithelial cells segregate and undergo two rounds of cell division. Following the first round of scale precursor division, one daughter cell dies and the other differentiates from the surrounding epithelia, becomes polyploid and increases in size. The surviving

Figure 1



Wing scale ontogeny in *P. coenia*. **(a–h)** Confocal micrographs of wing imaginal discs either stained with (a,d–f,h) rhodamine–phalloidin, which reveals cell outlines of developing tissues, (b) TO-PRO, a DNA dye, or (c) acridine orange, which labels dying cells, or (g) that were from a butterfly infected with a sindbis virus expressing green fluorescent protein (D.L.L., unpublished). (a) Fifth instar wing imaginal disc epithelial cells are undifferentiated. (b) At 15 h after pupation (AP), enlarged scale precursor cells (in the more apical plane; pink) appear to be dividing. The arrow indicates a mitotic figure. Two focal planes are shown, the more apical plane is colored purple and the basal one is green. White indicates staining in both focal planes. (c) At 17 h AP, cell death is visible by acridine orange staining, consistent with cell death reported in moths [12]. (d) By 24 h AP, the surviving scale lineage of cells have segregated and enlarged. (e) Between 24–26 h AP, some of the differentiated cells have begun to divide into scale-secreting and socket-secreting cells. Daughters of cell divisions are marked with asterisks. (f) By 28 h AP, more scale-secreting and socket-secreting cells are evident. Two focal planes are shown, colored as in (b). Socket cells are marked with arrowheads. (g) By 30 h AP, division of these differentiated cells into socket-building and scale-building cells is clearly visible. Daughters of this division are indicated by arrows. The arrowhead indicates a scale precursor cell daughter that has not yet divided. (h) By 72 h AP, scales are being secreted through their sockets. Two focal planes are shown, colored as in (b). **(i)** High magnification photograph of scales on the wing of a newly emerged butterfly. (a,d–i) Distal is to the right; (b,c) distal is at the top.

daughters divide into socket-building and scale-building cells. The first round of cell division in the butterfly *Precis coenia* appears to occur at 15 hours after pupation (AP) (Figure 1b). As predicted by Stossberg's [12] observations in moth wings, massive cell death is detectable in the wings of *P. coenia* at 17 hours AP (Figure 1c). The surviving cells are arranged in rows along the anteroposterior axis and are spaced apart along the proximodistal axis (Figure 1d). A second round of cell division, oriented along the proximodistal axis, gives rise to socket-building and scale-building cells (Figure 1e–g). Subsequently, scales grow through the sockets (Figure 1h), and eventually develop various forms of microarchitecture and pigmentation (Figure 1i).

The sequence of development of the structural scales of a butterfly is very similar to that of the sensory bristles of *Drosophila*, in which sensory mother cells (SMCs) segregate from the surrounding epithelial cells and undergo two rounds of cell division [16,17]. One SMC daughter divides to give rise to a neuron and glia, the other daughter cell divides to produce a socket and a bristle-building cell, which then grows through the socket. If scales and bristles are in fact homologous structures, then the non-innervation of scales is consistent with the programmed cell death of the basal daughter cell of the putative scale precursor cell; in *Drosophila* bristle development, the equivalent cell would survive to produce the neuron and glia [12].

In *Drosophila*, the *achaete-scute* (AS-C) genes promote neural precursor formation [18–23]; *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) promote the formation of overlapping sets of neuroblasts in the embryonic central nervous system (CNS), while in the epidermis, *ac* and *sc* are the two principal genes that promote the development of external sensory organs [24]. Each AS-C gene encodes a basic helix-loop-helix (bHLH) transcription factor and *ac*, *sc*, and *l'sc* are first expressed in cell clusters that usually resolve to a single precursor that enlarges and segregates beneath the epidermis. Once the expression of *ac*, *sc*, and *l'sc* is turned off, neural precursors express another AS-C gene, *asense* (*ase*), before they first divide and in the daughter cells produced by the first division [23,25].

Given the similarities between the cell biological events that promote neural precursor and scale cell formation and the requirement for AS-C genes to promote neural precursor formation in *Drosophila*, we speculated that AS-C genes might promote Lepidopteran scale cell development. In order to address this issue, we cloned an AS-C gene from *P. coenia* and determined its spatial and temporal pattern of expression. We found that scale precursors express the AS-C homolog, indicating that common genetic processes underlie the similarities between scales and bristles, and we conclude that Lepidopteran scales and insect sensory bristles are homologous structures.

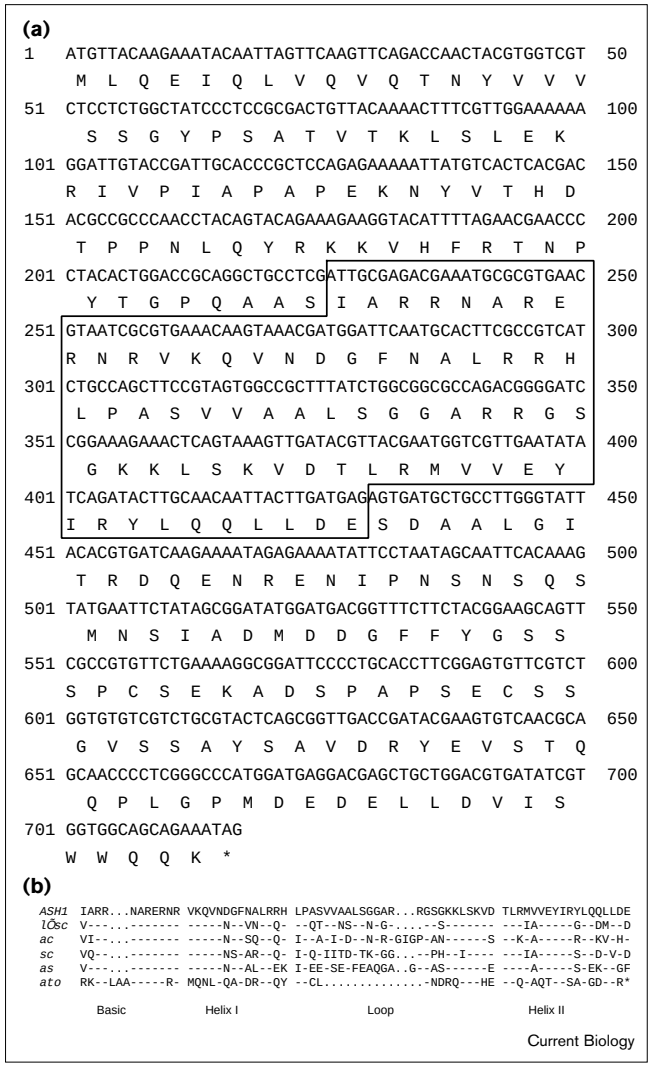
Results and discussion

Cloning of a butterfly AS-C homolog

A 162 bp fragment of a *P. coenia* AS-C gene homolog was isolated from butterfly genomic DNA using a degenerate polymerase chain reaction (PCR) method with primers that recognize conserved sequences in the basic and second helical domains of AS-C genes [26]. The fragment was used to screen a *P. coenia* embryonic cDNA lambda library. A partial cDNA was isolated as a single clone and found to contain a predicted open reading frame (ORF) coding for 167 amino acids, including a bHLH domain characteristic of all AS-C proteins. The embryonic cDNA library was rescreened using the partial cDNA fragment as a probe, and two additional positive plaques were isolated. Both of these appeared to be full-length 1.9 kb cDNAs corresponding to the same gene, which we have named *AS-C homolog-1* (*ASH1*; Genbank accession number AF071498) and which is predicted to encode a 238 amino-acid protein (Figure 2a).

Sequence alignment of the bHLH proteins encoded by the predicted ORF of *ASH1*, the four *Drosophila* AS-C genes and *Drosophila atonal* (*ato*) showed that *ASH1* is more similar to the AS-C genes than to *ato*, and that the bHLH domains of their encoded proteins are highly conserved (Figure 2b). Conservation within the loop is weaker, but the carboxy-terminal region of the loop shows high conservation. Although similarity outside the bHLH region is much lower, the butterfly homolog also shares amino-acid identity with *Drosophila* AS-C proteins at the carboxyl terminus. Our phylogenetic analysis of the bHLH domains encoded by *ASH1*, the *Drosophila* AS-C genes, and other known AS-C gene homologs, revealed that *ASH1* is no more closely related to any one *Drosophila* AS-C gene than to any other (data not shown). Phylogenetic analysis of AS-C genes is difficult, however, because of the short length of the conserved sequences and the large number of amino-acid changes within these regions. The unresolved relationship of butterfly and *Drosophila* AS-C genes might be explained by a high rate of nucleotide replacement in these genes during evolution, or a scenario in which the four *Drosophila* AS-C genes arose from duplications of an ancestral gene, or pair of genes, more recently than the divergence of the butterfly and *Drosophila* lineages.

Figure 2

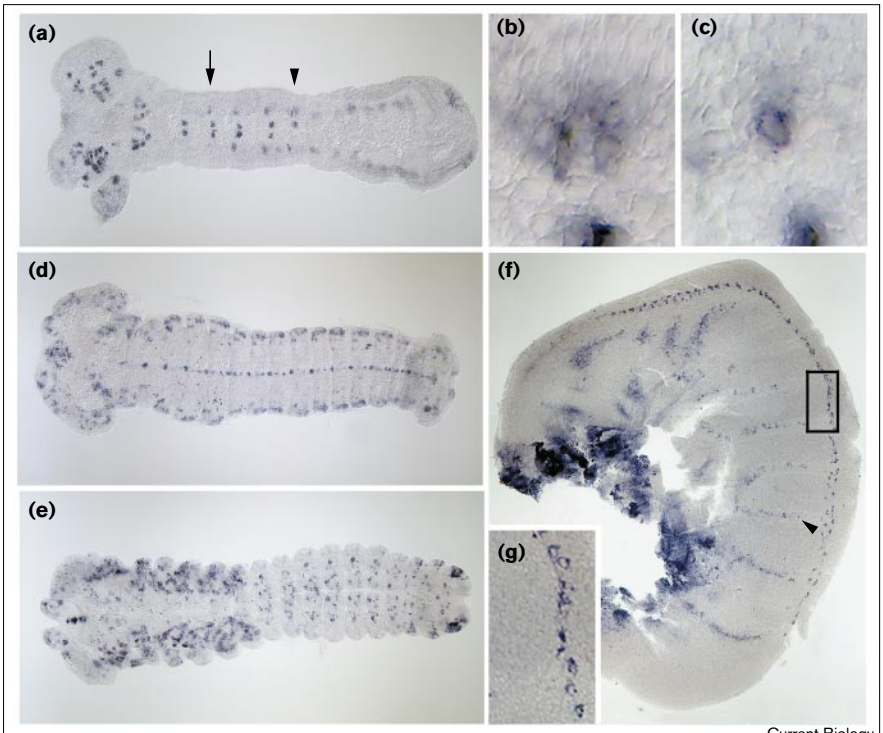


Predicted open reading frame of *ASH1* and its alignment to *Drosophila* bHLH proteins. **(a)** Nucleotide sequence and predicted amino-acid sequence of *ASH1*. **(b)** Alignment of the proteins encoded by the region of *ASH1* boxed in (a), the *Drosophila* AS-C genes and *ato*. *ASH1* is more similar to AS-C genes than to *ato*, although *ASH1* is not any more similar to any one AS-C gene in particular. The basic, helical, and loop regions are labeled. Dashes indicate sequence identity with the protein encoded by *ASH1* and dots indicate sequence gaps. Asterisks are stop codons.

Embryonic and larval expression of *ASH1* is similar to that of *Drosophila* AS-C genes

In order to elucidate the role of *ASH1* during butterfly development, we examined its spatiotemporal expression pattern during embryonic, larval, and pupal development using RNA *in situ* hybridization. *ASH1* expression showed a dynamic pattern in the embryonic nervous system. At the stage defined as ≈10% of embryogenesis, *ASH1* was expressed in one to three enlarged epidermal cells just lateral to the ventral midline, and in more lateral epidermal cells of the peripheral nervous system (PNS; Figure 3a). During this stage, in younger, more posterior segments, *ASH1* was expressed in what appear to be proneural clusters in the epidermis (Figure 3a, arrowhead and 3b). In more mature, anterior segments, this expression was refined to a single, selected putative neural precursor cell (Figure 3a, arrow and 3c). At ≈20% of embryogenesis, *ASH1* was expressed in the CNS in three cells per abdominal and thoracic segment, and in lateral clusters of enlarged epidermal cells both in these segments and in developing limb buds (Figure 3d). At ≈30%

Figure 3



Embryonic and larval expression of *ASH1*. **(a)** At ≈10% of embryogenesis (defined in [32]), *ASH1* is expressed in groups of one to three enlarged cells in the CNS flanking the ventral midline, and more laterally in epidermal cells in the PNS. Proneural clusters in the PNS are evident in younger, more posterior segments; arrowhead in (a) and shown at higher magnification in **(b)**. These clusters resolve to a single, selected neural precursor cell in more mature, anterior segments; arrow in (a) and shown at higher magnification in **(c)**. **(d)** At ≈20% of embryogenesis, *ASH1* is expressed in the CNS in three cells per segment located in the ventral midline, and in clusters of lateral epidermal cells of the PNS. **(e)** At ≈30% of embryogenesis, *ASH1* is no longer expressed in the CNS in trunk segments, but is expressed in enlarged cells in the PNS in abdominal segments and in developing legs. **(f)** In fifth instar wing imaginal discs, *ASH1* is expressed in enlarged cells along the wing margin – boxed region, shown at a higher magnification in **(g)** – and along the trachea (arrowhead). The positions of these cells predict the positions of sensory structures in the adult wing. (a–e) Embryos are mounted ventral side up and with anterior to the left; (f,g) the wing imaginal disc is mounted so that anterior is up and distal is to the right.

of embryogenesis, *ASH1* expression in the ventral midline in trunk segments had ceased, but *ASH1* was still expressed in the epidermis (Figure 3e). Overall, the embryonic expression of *ASH1* in *P. coenia* was very similar to that in *Drosophila*, in that *ASH1* was expressed in clusters of epidermal cells that resolved to a single cell later in development. This indicates that the early function of *ASH1* in butterflies is probably conserved with the proneural function of AS-C genes in flies.

A possible role for *ASH1* during fifth instar wing imaginal disc development was also investigated. During the last larval stage of *P. coenia*, epithelial cells in the wing imaginal disc have not begun to differentiate into scale-forming cells, and all cells are of uniform size and shape (Figure 1a). Progressive tracheal invasion of wing imaginal discs is an indication of their increasing age. Early in fifth instar development, when trachea had not yet invaded the wing disc, no *ASH1* message was detected. During the early-fifth to mid-fifth instar stage, when the trachea had almost reached the wing margin, *ASH1* was expressed along most of the dorsoventral boundary, including both the anterior and posterior margins (Figure 3f,g), and in cells along all trachea in both the forewings and hindwings (Figure 3f, arrowhead). The expression at this stage correlates with the position of innervated sensory scales [3], and indicates a likely function for *ASH1* in determination of the cells that give rise to these sensory structures. Later in the last larval stage, the number of *ASH1*-expressing cells decreased, most markedly in the posterior of the wing disc. *ASH1* was still expressed along the anterior wing margin and along the trachea, however. At the latest stages of the last larval instar, no *ASH1* staining was detected in wing discs (data not shown).

As with its embryonic expression, the larval expression pattern of *ASH1* is similar to that of AS-C genes in *Drosophila*. In third instar *Drosophila* wing imaginal discs, both *ac* and *sc* are expressed along the dorsoventral boundary and vein L3. In *Drosophila*, AS-C genes are not expressed in the posterior region of the wing, whereas *ASH1* is expressed along the entire wing margin in *P. coenia*. Regions of AS-C gene expression in *Drosophila* and *P. coenia* predict the positions of adult sensory structures and, hence, the expression of *ASH1* along the future wing margin and veins during *P. coenia* larval development suggests a conserved role for AS-C genes in determining cells that give rise to wing sensory structures.

ASH1 is expressed in scale-forming cells during pupation

In contrast to what is observed in *Drosophila* for AS-C genes, *ASH1* is expressed in cell types that differ between

larval and pupal stages. *In situ* hybridization is not possible before 24 hours AP so we could not survey *ASH1* expression during the time in which the first scale precursors are presumed to form. At 24 hours AP, *ASH1* was expressed in larger cells that had segregated from the surrounding epithelial cells and are present in the entire wing blade region. These cells are arranged in evenly spaced rows along the anteroposterior axis, predicting the arrangement of scales in the adult wing (compare Figure 1h and Figure 4a). The cells expressing *ASH1* are part of a structural (non-innervated) scale-forming cell lineage (Figure 4a), representing about one out of every ten epidermal cells.

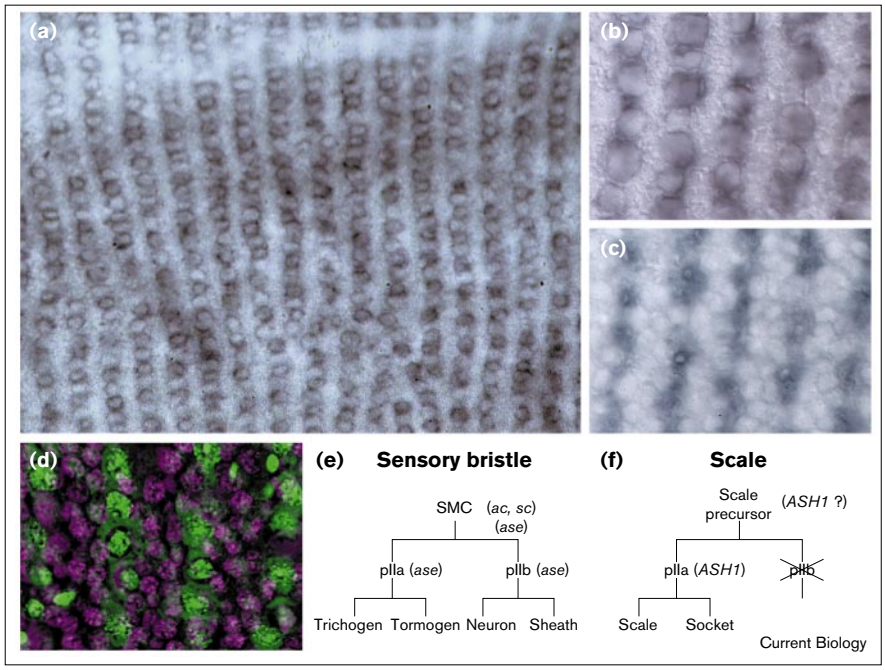
The identity of the *ASH1*-expressing cells was deduced by following their fate. These cells appear to be products of scale precursor cell divisions that occur at 15 hours AP (Figure 1b). Within two to four hours after *ASH1* expression is observed, these larger cells undergo a division that is oriented along the proximodistal axis (Figure 1e–g). These two daughters then differentiate as a socket and scale (Figure 1h). These observations, and previous studies of moth scale development [12], suggest that the *ASH1*-expressing cells are the equivalent of the pIIa cell in the sensory organ lineage (Figure 4e,f). The cells expressing *ASH1* thus appear to be the surviving daughters of the scale precursor cell (Figure 4f) and share several characteristics with *Drosophila* SMCs in that they express an AS-C gene, enlarge, and segregate from an epithelium and undergo a differentiative division. It is very likely, then, that *ASH1* plays a similar role in determining the fate of these cells as AS-C genes do in determining SMCs in *Drosophila*.

Conclusions

We have identified an AS-C gene homolog from *P. coenia* which is equally related to all of the *Drosophila* AS-C genes. The lack of clear orthology between *ASH1* and any individual *Drosophila* AS-C gene indicates either that these gene sequences are evolving rapidly, or that duplications of one or two AS-C gene homologs occurred in ancestors of the winged insects during the evolution of the *Drosophila* lineage, after its split from the Lepidopteran lineage.

Embryonic *ASH1* expression patterns in the CNS and epidermis suggest that *ASH1* plays roles similar to those of AS-C genes during *Drosophila* embryogenesis. *ASH1* expression, in what appear to be proneural clusters, and its subsequent restriction to a single cell, is consistent with a role in promoting the initial events of neural precursor selection and formation in *P. coenia*, as *ac*, *sc*, and *l'sc* do in *Drosophila*. Expression of *ASH1* in larval wing imaginal

Figure 4



Expression of *ASH1* in scale precursors in pupal wing discs. **(a)** At 24 h AP, *ASH1* is expressed in segregated, differentiated cells of the scale cell lineage. The nuclei of these cells occupy a very large proportion of the cell volume, so the *ASH1* staining in the cytoplasm appears as a cell outline. *ASH1*-expressing cells are arranged in rows along the anteroposterior axis of the wing, and *ASH1* expression predicts the arrangement of scales in adult butterfly wings. **(b)** A higher magnification view of *ASH1* expression at the same stage as (a) at the focal plane of differentiated cells. **(c)** Same magnification of the area depicted in (b), but at the focal plane of undifferentiated epithelial cells. This shows more clearly that *ASH1* is exclusively expressed in the enlarged cells of the scale cell lineage. **(d)** Confocal micrograph of a propidium-iodide-stained 24 h AP wing imaginal disc. Two focal planes, represented by different colors, are shown. Purple is more basal, while green is more apical. The nuclei of cells of the scale cell lineage project apically above undifferentiated epithelial cells. The siblings of the scale-forming cells are not visible, so we presume that they have died by this developmental stage. **(e)** The cell lineage of a *Drosophila* sensory bristle. The SMC first expresses *ac* and *sc*, and subsequently *ase*. Daughters of the SMC, pIIa and pIIb both express *ase*; pIIa divides into trichogen and tormogen, while pIIb divides into neuron and sheath cells. **(f)** The cell lineage of *P. coenia* scales. A scale precursor cell (equivalent to the SMC in *Drosophila*) undergoes a cell division. One of its daughters dies, while the surviving daughter (pIIa) divides again to become socket-secreting and scale-secreting cells. The surviving daughter of the scale precursor expresses *ASH1*. It has not been determined if the scale precursor cell also expresses *ASH1*.

discs in regions where innervated sensory structures are located in adult wings, as in *Drosophila*, indicates that AS-C genes are also likely to be used in the development of all insect sensory bristles.

The expression of *ASH1* in rows of progenitors of the scale and socket cells suggests a role for *ASH1* in scale development. Coupled with the embryonic and larval patterns of *ASH1*, it appears that *ASH1* is playing more roles than any individual AS-C gene in *Drosophila*. For example, *ac* and *sc* are expressed in proneural clusters and neural precursors, but switch off before precursor division, whereas *ase* is expressed in both precursors and their daughters. *ASH1* in butterflies seems to exhibit expression traits of all four *Drosophila* AS-C genes, and might have both the proneural role of *ac* and the neural precursor role of *ase*. It is possible that if duplication of AS-C genes occurred after the Lepidopteran–Dipteran split, differential regulation of AS-C genes led to their divergent roles during *Drosophila* neurogenesis.

Although the domains of AS-C gene expression in pupal wings differ between *P. coenia* and *Drosophila*, the function of AS-C genes in the segregation of cells from an epithelium appears to have been conserved during the evolution of Lepidopteran scale-covered wings. We propose that during the evolution of the Amphiesmenoptera (which include the Trichoptera — a sister taxon to the Lepidoptera characterized by wings covered by non-innervated bristles — and the Lepidoptera), the basic function of AS-C genes in precursor cell segregation was maintained in the development of non-innervated bristles. Genetic changes in the control of cell lineage, downstream of AS-C genes, and in the upstream spatial regulation of AS-C genes must have occurred. That is, wing sensory structures lost their associated neuron and glial cell, and, through spatial changes of AS-C gene regulation, bristles covered the wings entirely. Subsequent to these events, during the evolution of the Lepidopteran lineage, genes downstream of AS-C genes that control cytoarchitecture and pigmentation might have changed as bristles were modified into scales. The use of AS-C genes in butterfly scales and *Drosophila* sensory bristles leads us to conclude that the morphological and developmental similarities between these structures is due to the use of similar genetic programs, and that Lepidopteran scales and insect sensory bristles are homologous structures.

Materials and methods

Staining procedures and labeling using green fluorescent protein

For staining using TO-PRO, wing discs were fixed as described previously [27] and incubated in 10^{-6} M TO-PRO (Molecular Probes, Inc.) in 50% glycerol, 100 mM Tris (pH 7.4), and 150 mM NaCl for 90 min. For phalloidin staining, pupal wing discs were fixed as described previously [27], and mounted in 20% glycerol, 100 mM Tris (pH 7.4), and a 1:10 dilution of a 3.3 μM stock solution of rhodamine–phalloidin (Molecular Probes, Inc.). For staining using acridine orange, pupal wings were dissected in 1.6×10^{-6} acridine orange in cold PBS, and immediately mounted and photographed. To label cells with green fluorescent protein, fresh pupa were injected with a Sindbis virus expressing green fluorescent protein (D.L.L., unpublished). At 30 h AP, wings were dissected and fixed.

Cloning and sequencing of ASH1

Degenerate PCR on 700 ng butterfly genomic DNA was performed using primers and conditions described by Johnson *et al.* [26]. A 162 bp fragment amplified by this approach was cloned into pBluescript (Strategene) and manually sequenced by the method of Sanger *et al.* [28]. This fragment was used to screen 5×10^5 clones from a butterfly embryonic cDNA library at low stringency [27], upon confirmation that the PCR fragment encoded part of a bHLH gene. DNA preparation and subcloning of the positive clone isolated in this screen were performed as described by Sambrook *et al.* [29]. The single, partial cDNA clone was sequenced, confirmed to encode the amino terminus and the bHLH domain of an AS-C homolog, and subsequently used to screen 5×10^5 clones from the cDNA library. For positive plaques, primers targeted to phage lambda arms were used to amplify inserts directly from phage elutions, the amplified products were cloned into pCR2.1 (Invitrogen) and sequenced.

Phylogenetic analysis

Gene trees were constructed using PHYLIP (Phylogeny Inference Package v3.572c) and PAUP (Phylogenetic Analysis Using Parsimony v3.1.1). As many as 71 amino acids of vertebrate (mouse, rat, chicken, zebrafish, and *Xenopus*), insect (*P. coenia*, *Drosophila melanogaster*, and *Tribolium*) and hydra AS-C protein sequences spanning the bHLH domains were aligned as shown in Figure 2b. The bHLH domains encoded by *myoD*, *nau*, and *ato* were used as outgroups and aligned similarly. In PHYLIP, the Seqboot program resampled each data set 100 times for bootstrap analysis, the Protdist program calculated distances using a PAM–Dayhoff distance matrix [30], and the Fitch program used the Fitch–Margoliash least-squares model to search for the best trees. Maximum parsimony trees were constructed using both the Protpars program from PHYLIP and using PAUP. Bootstrap values in PHYLIP were calculated with the Consense program.

In situ *hybridization*

Butterfly embryos and fifth instar and pupal wing imaginal discs were probed with a digoxigenin-labeled anti-sense RNA probe, using methods adapted from Hauptman and Gerster [31] and Carroll *et al.* [27]. The partial cDNA clone encoding the amino terminus and entire bHLH region of ASH1, isolated from the first library screen, was used as the template. For all developmental stages, sense-strand controls were performed, and no staining was observed.

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